Quantitative determination of free fatty acid in bile and gallstones from Basra cholelithiasis by Gas Chromatography

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ABSTRACT

A simple method for the gas chromatographic quantitation of fatty acids in the bile and gallstone is described where fatty acids are subjected to methylation derivative. 37 patients from cholelithiasis, admitted at Basra general hospital, they were 17 patients with gallbladder cholesterol stones, 14 patients with interahcpatic bile duct pigment stones whereas 6 patients with extrahepatic bile duct cholesterol stones .Gallbladder bile was collected from 37cholelithiasis patients. In our study, analyzed them quantity fatty acids in different stones.

The total free fatty acids and fatty acid contents of bile and cholesterol stones samples in the intrahepatic cholesterol were markedly higher than individuals in samples from bile and pigment stones($p \leq 0.05$).The ratio of free saturated to free unsaturated fatty acids were highest in the gallstones, bile for intrahepatic cholesterol ($p \leq 0.05$).

INTRODUCTION

Gallstone disease remains a serious health concern for human beings a effecting millions of people through out the world [1].in Iraq recent years has seen an increasing trend in the number of gallstone cases in southern Iraq[2].Gallstones have been classified into three classes based upon their physico-chemical characteristics, cholesterol stone, pigment stone, and mixed stones.The cholesterol stones are formed in gallbladder, while pigment stones are mostly formed in bile canliculi of liver and mixed stones to grow entire biliary tract and settle down in gallbladder.etiologically, many factors including age, bacterial infection, malnutrition, impaired intestinal barrier function may contribute to the cholelithiasis [3].Fatty acids exist in the body free (unesterified) or as fatty acylesters in more complex molecules, these compounds contains may be standard chain or may contain one or more double bonds at specific positions, or they may be fully saturated. The physical and chemical properties of any chain from free fatty acid depend on the composition of fatty acid bounding to make lipids. a large proportion of the fatty acids used by body is supplied by the diet, ingested lipids are emulsified in the small intestine by means of bile that coming from the gallbladder and liver.

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This emulsification is a precondition for hydrolysis of the various lipid esters. In a man are mostly straight chain mono carboxylic acids with an even number of carbon atom between (4to more than 20) mainly derived from dietary triglycerides(4). To date, the reports on fatty acid metabolism in gallstone remain few, we are analyzed the quantity of fatty acid in different gallstone types by the a simple method for the gas –liquid chromatography that described where free fatty acids are subjected to methylester derivative, this process were found to be highly reproducible and gives results comparable with other well established method (5).

MATERIALS AND METHODS

Thirty seven patients from suffering cholelithiasis ,admitted at Basra general hospital, they were 17 patients with gallbladder cholesterol stones, 14 patients with interahcpatic bile duct pigment stones whereas 6 patients with extra hepatic bile duct cholesterol stones .Gallbladder bile was collected from 37cholelithiasis patients during the operation with sterile syringe . The gallstones were classified according to the quantitative analysis of gallstone were the stone having more than 70% cholesterol of there dry weight there is cholesterol stone (CS) , while the stone having less than 30% cholesterol dry weight this is pigment stone (PS). The stone collected was washed with dieionized-distilled water 2-3 times until washing became clear, if the specimen consisted of multiple stones, i.e one to the three were selected randomly for analysis all stones were pulverized to fine powder with mortar and pastel for 5 minutes, this process produced a fine homogeneous powder which was then stored in a sample tube, kept over silica jel in dark cabinet until for analyzed.

Gas chromatography

Japan shimadzu company model (G.C.9A) equipped with a flame ionization detector and an injector with a split/split less device for glass column was used in all separation. The chromatographic column (6m x 2.6mm) consisted of chemically bound fused silica 5% OV_1 (poly siloxane) on cromosrb W and helium gas was used as the carrier gas. The peak heights were used to calculate the fatty acids content.The fatty acids chosen for controlling for bile and gallstones samples were Butyric (4:0),Lauric (12:0),Palmitic(16:0),palmitoleic(16:1), Stearic(18:0),Oliec(18:1),Linoliec(18:2) ,Linolenic(18:3),Archidic(20:0),12hydroxy stearic acids(18:1).

Standard preparation

Stock solution of fatty acids standard (250 mg/2.5mL)were prepared in mixture of hexane, butyl alcohol solution (10:1).stock solution of internal standard was prepared by dissolving 0.1g of myristic acid in 10 mL of mixture hexane and butyl alcohol solution(10:1).

Sample preparation

Freeze-bile and gallstone powder for each (15mg) to which (20µg)myristic acid was add and heated with 5 mL from hexane and butyl alcohol solution (2:1) at 65C° for 1-hour .after centrifugation the supernatant was collected and the residual was a gain extracted with hexane ,butyl alcohol solution . Then mixtures were evaporated to dryness (content fatty acids).20µg of fatty acid samples from bile and gallstones which added to 50 mL of concentrated hydrochloric acid, the content were heated at 60C° in boiling water for 4 hours . After boiling take 1 mL of this solution as added to 2 mL for the main reagent

(0.1mL acetylchlorid add to 30 mL of methanol),that was to make the methylation derivatives .A volume of 1µL of the filtrate was analyzed by gas chromatography.Suitable volume after derivative of stock solution and 1mL of internal standard solution were used for preparation of working standards (15, 25, 50,100,150,200 mg/mL).

RESULTS

In an extension of our method, we have found that a when mixture of several biliary fatty acids were subjected to the conditions of ester and injected into the gas chromatography each class of these compounds were resolved from the other, figure (1) showed the chromatogram obtained by using this method to analyzed fatty acids methylation in bile and gallstone samples in our study. The retention time of standard fatty acids were obtained from chromatograms, compare with the value of retention time for the extracted individual fatty acids samples ,table (1),indicated the value of retention time of esterified derivatives of a numbers fatty acids in the present study. To quantify analysis for amount of each of the fatty acid methyl estered [FAMES]in our sample , they would compare the sample peak areas with the standard peak areas of standard fatty acids ,however ,the quantities of fatty acid derivatives were determined in all samples by comparison of peak areas with those of know amounts of appropriate reference compounds in a rang where a linear response was obtained ,table (2) showed the methylation derivatives concentration of the several fatty acids in bile and gallstones. Comparsion of the content of intrahepatic cholesterol stones and pigment stones were (310.09±49.7 ,55.59±7.71) respectively, that appeared significantly different values (t=4.05.$p \leq 0.05$).while the bile for intrahepatic cholesterol stone and bile of pigment stone the content of free fatty acids were (402.75±71.8 , 96.67±5.50)respectively table 2,figure 2.

Ratio of free saturated to free unsaturated fatty acids (S/U)

The S/U values in the gallstones were (8.6±3.1) for intrahepatic cholesterol and 4.8±1.5 for pigment stones (t=2.121, $p \leq 0.05$). In the bile which taken from patients, the values were 3.1±2.1 for intrahepatic cholesterol and 1.3±0.4 for pigment stones. Which were significantly higher than 1.3±04 for black and 1.0±0.3 for cholesterol (t=3.2, 3.309, 2.73 and 2.5) respectively.

DISCUSSION

As co-investigators in clinical studies to determine the effects of increased amounts of chain fatty acid(CFA) in the every human sample , our asked to develop a simple , rapid , quantitative method for (CFA) in a variety of matrices are available[6] , including extraction[7] , several types of distillation and paper chromatography[8] .However, extensive sample handling and preparation make them unsuitable for our purposes. We have developed an appropriate method.In our study , the CFA were separated and quantity was determined by gas chromatography ,is the most current method in fatty acids analysis [9],this method has many advantages, such as high degree of separation of components with high structural similarity , it is also capable to analyze very little amount of samples (0.1-50µL) [10], in contrast ,the methods of Nakayma and Jihnston has applied the use of silicic acid column chromatography for complete analysis bile fatty acids[11] and the methods Greber and rose-Gottlieb, to used standard extraction method for quantitative determination of short chain fatty acid in human gall stone,

those method is –rather time-consuming and requires large amount of sample[12,13] .DiCor-cia and authors noted that higher concentration would overload of measurement coming peak broadening and effecting the quality of analysts[14] .In order to be more volatile,usually acids analyzed by gas chromatography are first esterified , with appropriate better resolutions between individual fatty acids which may be obtained and more complex mixture of fatty acids may also be resolved by method ester derivatization .In another method , prepared the n-butylester –acetate derivatives of fatty acids in plasma . Recently, many worker showed to have several drawbacks of acetate-n-butylester derivatives of bile and fatty acids including reduced sensitivity of acetate derivatives and increased retention time [15].A polyester liquid phase, 5%OV_1 developed by Ottenstein *etal*,provided sharp, complete separation of all the acids. Total analysis time (3min) was half of that for either of the other columns tested, however, no data were presented described the linearity on this packing [16]. In dealing, as we were with biological samples we expected that the rang of concentrations encountered would be rather broad to encompass both normal and abnormal concentration of fatty acids chain ,thus, it was necessary to verify the linear rang each acid on 5% OV_1 our decided that addition of a suitable internal standard would eliminate these variable parameters ,that use of myristic acid as internal standard, this finding agreement with Ackman had reported for used myristic acid and cyclohexane as internal standard[17] .

In this study, from Iraq southern the pigment stones make up 35% of cases of cholilethiasis, especially intrahepatic stones, which are almost, pigment stones. Maki *etal* proposed that bacteriogenic β–glucuronidase can catabolize the combined type of bilirubin to the free type,which further precipitates to appearance pigment stones[18].In the present study 85% of free fatty acids in intrahepatic cholesterol were more than in any other kind of stones, according to this results were nearly compatible with the reported by Wang W *etal* that showed this phenomena [19] while not agreement with the another studies that employed in China cholilethiasis that shower the large amount of fatty acids of free type in intrahepatic pigment stones [20]. The fatty acids in the stones originate from the phospholipids in bile juice ,our result also indicate that formation of intrahepatic stones results from the precipitation if free fatty acids produced by dissolution of phospholipids under the effect of phospholipase A_1[21] .This is different from black and gallbladder cholesterol stones . It was believed in the past that the enzyme involved was phospholipase A_2, coming from pancreatic reflux. But this enzyme mainly catalyzes the Sn-2bond of unsaturated fatty acids, such as oleic and linoliec acids .Our experiments showed that there are more free saturated than unsaturated fatty acids in intrahepatic and extrahepatic cholesterol stones that is to say ,the level of palmitate and stearic acids are increased ,this indicates that the action of phospholipase A_1 is more important than that of phospholipase A_2[22].Nakano *etal*, measured the amount of free fatty acids and the activity of phospholipase in the bile juice infected or uninfected with bacteria .Variety of bacteria in bile juice have an active function of producing phospholipase A_1 and phospholipase A_2, this illustrates the important role of fatty acids in the formation of intrahepatic and

extrahepatic for different types gallstones [23].Phospholipase especially phospholipase A, is chief in the dissolution and precipition of fatty acids [24]. In addition, raise the concentration of several free fatty acids as (palmitic, lauric,stearic acids) in the hepatic bile reducing cholesterol solubilizing capacity ,this probably causes cholesterol gallstone formation[25].

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Figure 1. GC chromatogram of fatty acids present in bile and gallstones from cholilethaisis patients,Peak identification;1-Butyric,2-Lauric,3-Palimtoleic,4-Palmitic,5-Myristic,6-Oleic,7-Stearic,8 Linoleic ,9- Linolenic 10-Arichidic,11-12hydroxy stearic acids.

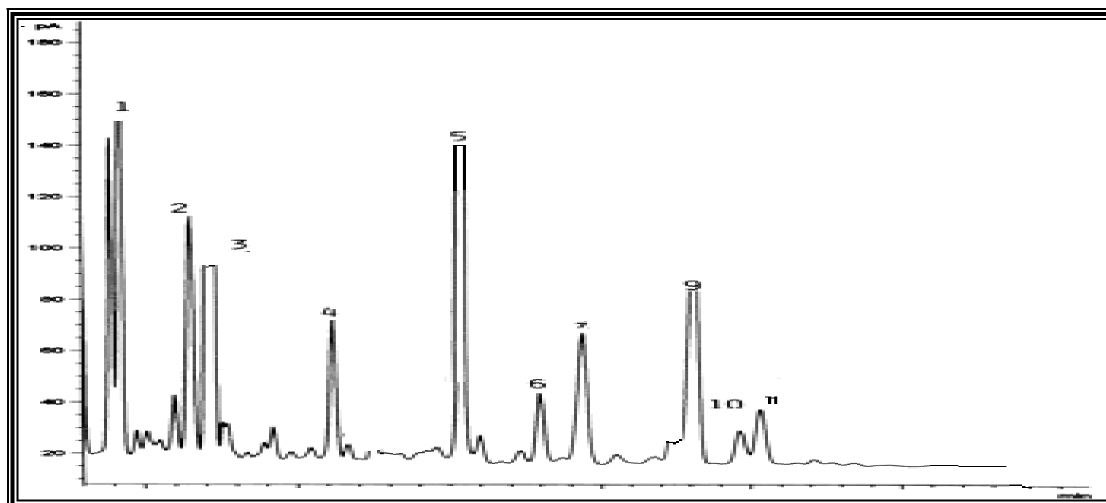


Table 1. GLC retention times of methylation derivatives esters of fatty acids, on 5% OV_1 column.

Fatty acids derivatives	Retention time (min)	Fatty acids derivatives	Retention time (min)
Butyric acid	0.223	**Linoleic acid**	0.355
Lauric acid	0.291	**Linoleinic acid**	0.392
Palmitic acid	0.356	**Archidic acid**	0.433
Palmitoleic acid	0.352	**12-Hydroxy stearic acid**	0.655
Stearic acid	0.389	**N-Myristic acid***	0. 400
Oleic acid	0.410		

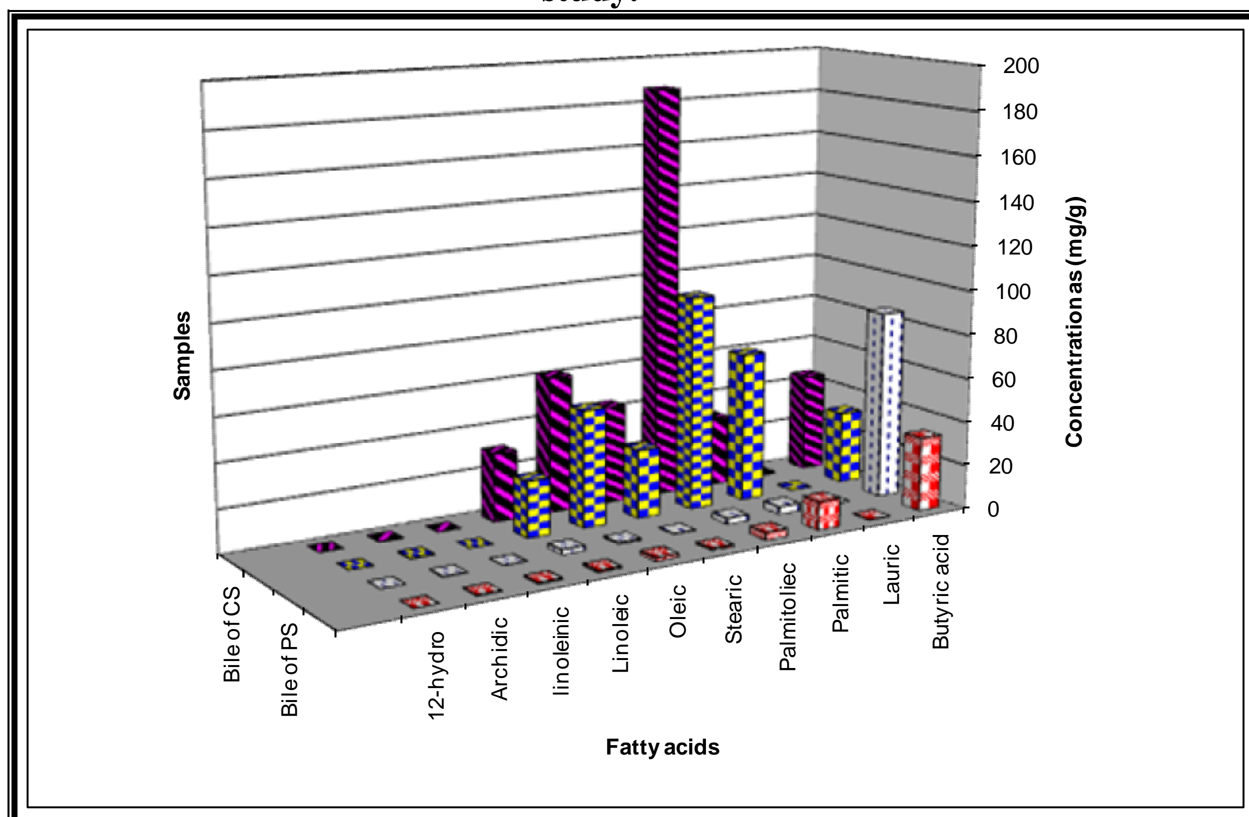
*** Retention time (min) are expressed relative to that of the N-Myrstic methyl ester**

Table 2, Contents of free fatty acids (mg/g) in gallstone and bile from cholilethaisis patients

Fatty acids(mg/g)	Intrahepatic cholesterol stones		Intrahepatic pigment stones	
	Gallstones	Biles	Gallstone	Biles
Butyric acid	33.1±8.43	45±10.2	33.7±7.21	85.18±17.89
Lauric acid	0.09±0.01	0.72±0.05	0.32±0.1	0.1±0.02
Palmitic acid	67.8±5.19	32.1±6.3	12.8±3.72	3.5±1.12
Palmitoleic acid	97.1±27.01	187±20.6	3.5±0.78	2.68±0.24
Stearic acid	31±5.01	44.5±6.02	0.96±0.08	023±0.02
Oleic acid	53±7.71	62.01±7.08	1.69±0.11	0.9±0.15
Linoleic acid	25.33±5.5	31±13.20	0.83±0.15	2.21±0.15
Linoleinic acid	0.03±0.01	0.0	0.6±0.098	011±0.04
Archidic acid	0.35±0.1	0.38±0.07	0.35±0.1	0.27±0.08
12-Hydroxy stearic acid	0.02±0.001	0.05±0.019	0.0	0.0

All data were expressed as the Mean± SD,* $p \leq 0.05$

Fig2:- Distribution of fatty acids concentration in bile and gallstone patients for different gallston type in our study.



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تحديد نسبة الأحماض الدهنية الحرة في الصفراء و حصى المرارة لمرضى أستئصال المرارة من البضرة بطريقة كروموتوغرافيا الغاز

د. محمد عبد المنذر ، د. فائز الجميلي

الخلاصة

استخدمت تقنية كروماتوغرافيا الغاز Gas chromatographic لتعين الكمي لبعض الحوامض الدهنية في نماذج الصفراء والحصى المرارية التي جمعت بعد أجراء عملية استئصال المرارة من مراجعي مستشفى البصرة العام،شملت هذه الدراسة (٣٧) مصابا حيث كانوا موزعين (٢٣)مصابا من مرضى حصى الكوليستيرولية ،(١٤)مصابا من مرضى الحصى الصباغية مع نماذج الصفراء لجميع هؤلاء المصابين.النتائج التي تم الحصول عليها من خلال هذه التقنية بينت ارتفاعا في مستوى المحتوى الكلي للأحماض الدهنية في نماذج الصفراء والحصى الكوليستيرولية ،كذلك ارتفاع ذو فرق معنوي عالي في نسبة الأحماض الدهنية المشبعة من غير المشبعة في نماذج الصفراء والحصى الكوليستيرولية ومقارنتها مع نماذج الصفراء والحصى الصباغية ($p \leq 0.05$)